PECAM-1 phosphorylation and tissue factor expression in HUVECs exposed to uniform and disturbed pulsatile flow and chemical stimuli

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Introduction: **We analyzed the relationship between platelet endothelial cell adhesion molecule-1 (PECAM-1) activation and tissue factor (TF) RNA expression in human umbilical vein endothelial cells (HUVECs) exposed to mechanical and chemical stimulation.**

Methods: **Fifty percent confluent and 100% confluent HUVEC cultures were exposed to pulsatile forward flow, as a model for uniform flow, or pulsatile to-and-fro flow, as a model for disturbed flow, using a parallel-plate flow chamber system for up to 4 hours in the presence or absence of 4 U/mL thrombin. Protein lysates were immunoprecipitated for PECAM-1 and then immunoblotted with anti-phospho-tyrosine antibody. TF RNA expression was determined using quantitative reverse transcription polymerase chain reaction.**

Results: **HUVECs exposed to disturbed flow induced higher TF expression at 4 hours than HUVECs exposed to uniform flow in sparse cultures (16.8 ± 5.8 vs 5.1 ± 1.2; $P < .05$). HUVECs exposed to disturbed flow and thrombin induced higher TF RNA expression at 4 hours than cultures exposed to uniform flow and thrombin in both confluent (47.0 ± 6.0 vs 30.2 ± 4.9; $P < .05$) and sparse (72.3 ± 10.7 vs 49.8 ± 4.7; $P < .05$) cultures. In confluent HUVEC cultures, PECAM-1 is minimally phosphorylated by disturbed and uniform flow, while in sparse HUVEC cultures, PECAM-1 phosphorylation at 15 minutes is greater in both disturbed and uniform flow (2.0 ± 0.2 and 2.1 ± 0.4 respectively; $P < .05$). Thrombin treatment of static HUVECs exhibited greater PECAM-1 phosphorylation at 15 minutes in confluent compared with sparse cultures (3.0 ± 0.5 vs 2.3 ± 0.1; $P < .05$). PECAM-1 phosphorylation of HUVECs exposed to both flow and thrombin is significantly higher in sparse cultures compared with either flow or thrombin stimulation alone but was suppressed in confluent cultures.**

Conclusions: **The significantly higher TF RNA expression induced by disturbed flow and cell confluence indicates that suppression of PECAM-1 phosphorylation may be an important contributory mechanical signal pathway that promotes TF expression when HUVECs are exposed to disturbed flow. (J Vasc Surg 2015;61:481-8.)**

Clinical Relevance: **We demonstrate that cultured endothelial cells exposed to disturbed flow had more tissue factor (TF) expressed than those exposed to uniform flow. Since TF is found in areas of atherosclerosis, this presents an interesting correlation with the observation that disturbed flow is associated with atherogenesis. The finding of greater TF expressed by sparse human umbilical vein endothelial cell cultures may also contribute to our understanding of the mechanism by which areas of endothelial denudation are prone to intimal hyperplasia.**

In vivo, endothelial cells (ECs) are exposed not only to the substances that are in the blood but also to the complex mechanical forces generated by the circulation. Two distinct flow types are commonly found. The first, laminar flow, is unidirectional and typically found in large, straight regions of the arterial vasculature.[1] This flow is characterized by parallel layers of flow with little to no disturbance of movement between the layers. The second type of flow, disturbed flow, exhibits significant temporal and spatial gradients characterized by flow reversal and turbulence. Depending on the type of mechanical stimuli, ECs respond by altering cell function, which may result in pathology.[2-4] For instance, atherosclerotic lesion development corresponds to the site of the vessels that are subjected to disturbed flow and under conditions of low shear stress.[5-9] They include areas such as the lesser curve of the ascending aorta where shear stress is low, <1.5 N/m^2 in humans, but not in the descending aorta where there is uniform flow in the straight arterial segments and the shear is >1.5 N/m^2.

A prothrombogenic molecule, tissue factor (TF), has been implicated in inflammation in a variety of disease

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models and has been associated with atherosclerosis.[10,11] Although under physiological conditions TF is undetectable in ECs, several studies have shown a substantial increase in TF levels in atherosclerotic lesions.[12,13] Furthermore, in TF pathway inhibitor-deficient mice, significantly higher levels of atherosclerosis were observed in areas of disturbed flow, indicating that inhibition of TF activity may be an important atheroprotective mechanism.[14]

The mechanisms by which disturbed flow leads to atherosclerosis are not completely elucidated. Proteins commonly expressed by ECs during inflammatory responses and thrombogenesis can be found in great proportions in atherosclerotic plaques, and so inflammation and endothelial dysfunction may be key mechanisms. One putative proatherogentic protein found in ECs is platelet endothelial cell adhesion molecule-1 (PECAM-1).[15] PECAM-1 (CD31) has been characterized as a mechanoreceptor found at cell-cell junctions and thought to respond to the shear stress caused by blood flow.[16] PECAM-1 may have a proatherogenic role in the lesser curvature of the arch where disturbed flow predominates.[17] Furthermore, downregulation of PECAM-1 has been shown to stimulate thrombin-induced expression of TF by modulation of PI_3K activation.[18] The proposed working model for PECAM-1–initiated mechanosignal transduction is that mechanical force acts on PECAM-1 at the cell-cell adhesion and affects phosphorylation of PECAM-1 at the two tyrosine residues.[19-21] PECAM-1 in adjacent cells are thought to interact to work as mechanosensors upstream of the signal cascade. We previously reported that cell-to-cell contact was required for shear-stress–induced PECAM-1 phosphorylation when human umbilical vein endothelial cells (HUVECs) were exposed to orbital laminar shear.[22] Thus, we posit that the degree of confluence of ECs might affect PECAM-1 activation, leading to a different induction of TF expression under uniform or disturbed flow conditions.

Since PECAM-1 and TF are influenced by the presence of disturbed flow, and both are implicated atherosclerosis, the aim of this study was to assess the role of PECAM-1 in modulating hemodynamic and chemical stimuli that induce TF expression. Specifically, we analyzed PECAM-1 phosphorylation and TF RNA expression in HUVECs exposed to pulsatile disturbed flow and uniform flow in the presence or absence of thrombin, a chemical that also interacts with PECAM-1 and has been shown to induce TF expression in ECs.[18] To further define the role of PECAM-1, we investigated the impact of the degree of confluence of HUVECs exposed to hemodynamic force and chemical stimulation on TF expression. We hypothesized that due to less cell-cell contact in the nonconfluent state, PECAM-1 would be more susceptive to mechanical and chemical stimuli, and thus induce higher TF expression than in the 100% confluent state.

METHODS

Cell culture. Primary cultures of HUVECs were obtained from the laboratory of Dr Jordan Pober (Department of Pathology, Yale School of Medicine). Cells were cultured in 75-cm^2 flasks coated with gelatin with M-199 medium enriched with 20% fetal bovine serum (FBS; Gemini Bio-Products, West Sacramento, Calif), 10 μg/mL heparin, 50 μg/mL EC growth supplement (BD Biosciences, Bedford, Mass), and penicillin-streptomycin antibiotic combination (both 100 μg/mL), in a 5% CO_2 incubator at 37°C as previously described.[5,23,24] When cultures were 100% confluent, 0.25% trypsin ethylenediaminetetraacetic acid was added to detach them from the flasks, and HUVECs were seeded on fibronection (BD Biosciences) coated glass slides (75 × 38 mm: Fisher Scientific, Pittsburgh, Pa). The cells were seeded at either 4.0×10^6 cells/slide to attain 100% confluence (slide completely covered with cells with no room left for cells to grow) and termed as *confluent*, and at 2.0×10^6 cells/slide to achieve 50% confluence (roughly half the slide is covered with cells), and termed as *sparse*, and allowed to attach for 24 hours. Before experimentation, the degree of confluence of cultures was confirmed by microscopy.

Mechanical stress exposure. HUVECs were exposed to shear stress, utilizing a parallel-plate flow chamber system (Cytodyne, San Diego, Calif) as previously described.[5,23,25] The flow of the perfusion medium was regulated by a computer-controlled syringe pump (PHD Ultra Programmable; Harvard Apparatus, Holliston, Mass), which can be programmed to generate different flow profiles. The syringe pump contains a small step angle motor, which drives a screw that can push or pull back the syringe plunger. This allows both infusion and withdrawal of medium with an accuracy within 0.35% and reproducibility within 0.05%. To generate pulsatile forward flow (PFF) as a model of uniform flow, an automated switch clamp (Auto-Fill valve box; Harvard Apparatus) is placed between the syringe pump and the flow chamber and between the syringe pump and the culture medium reservoir, to allow unidirectional flow. Activation of both switch clamps is synchronous with the cycle. To generate pulsatile to-and-fro flow (TFF), as a model of disturbed flow, the flow chambers were directly attached to the flow loop circuit including the flow reservoir. The switch clamp is not used, and this enables culture medium in the flow chamber to be exchanged by every to-fro impulse. The flow regimens used were 60 cycles per minute (cpm) PFF (ie, a 0.5-second square wave forward ramp flow alternating with a 0.5 second of no flow) and 60 cpm TFF (ie, a 0.5-second square wave forward ramp flow alternating with 0.5-second square wave backward ramp flow), and the magnitude of the shear was kept constant at 14 dyne/cm^2. HUVECs not subjected to shear stress were kept in the incubator and served as the static control.

Chemical stress exposure under static and flow conditions. M199 culture medium with 1% FBS and 4 U/mL of thrombin (Sigma-Aldrich, St. Louis, Mo) was applied to static HUVEC cultures and to HUVEC cultures exposed to either disturbed or uniform flow and was present throughout the entire experiment. This dose of thrombin results in a reproducible stimulation of TF expression.[5,18,23,24,26] The reduction of FBS from 20% to

1% prior to experimentation and the relatively short time frame of exposure of HUVECs to flow, preclude any significant effect of proliferation. Experiments applying mechanical and chemical stress together were performed simultaneously with the experiments assessing only mechanical stress, as paired experiments.

Immunoprecipitation and immunoblot of phosphorylated PECAM-1. Cells were washed with phosphate-buffered saline and lysed in radio-immunoprecipitation assay lysis buffer (1% sodium dodecyl sulfate, 1-mM phenylmethylsulfonyl fluoride, and 2-mM sodium orthovanadate) supplemented with phosphatase inhibitor I and II cocktails (EMD4Bioscience, San Diego, Calif) and protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, Ind) at 4°C for 3 minutes. Insoluble fractions were separated by centrifugation at 15,000 × g for 25 minutes at 4° C. Aliquots of cell lysates (containing 20-μg proteins) were incubated overnight with rabbit anti-PECAM-1 (C-20, sc-1505-R; Santa Cruz Biotechnology, Santa Cruz, Calif) and 15 μL of Protein G-Agarose beads (Santa Cruz Biotechnology). The immunoprecipitates were washed three times with the lysis buffer and then eluted by boiling for 5 minutes in Laemmli sample buffer. The samples were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred onto polyvinylidene fluoride membrane, and probed with anti-phosphotyrosine-specific antibody (anti-Phosphotyrosine, Clone: 4G10; Milipore, Billerica, Mass). Tyrosine-phosphorylated PECAM-1 was detected using an enhanced chemiluminescence system. To ensure equal loading, membranes are stripped and reprobed with total antibodies (C-20, sc-1505-R; Santa Cruz Biotechnology), and phosphotyrosine band intensities are normalized to PECAM band intensities.

After the membranes were developed on X ray film, they were scanned using a PERFECTION 1660 scanner (Epson, Long Beach, Calif) and the bands quantitated with densitometry. Densitometric values were obtained using ImageJ public software (http://rsbweb.nih.gov/ij/index.html). The densitometric values were expressed as the fold change compared with the static control level.

TF RNA isolation and measurement. HUVEC total RNA was isolated using the RNeasy Mini kit (Qiagen Sciences, Germantown, Md) according to the manufacturer's instruction. RNA was reverse-transcribed into cDNA using the iScript reverse transcription kit (Bio-Rad Laboratories, Hercules, Calif), and polymerase chain reaction (PCR) was performed in a C1000 Thermal Cycler (Bio-Rad). TF messenger RNA (mRNA) levels were estimated by quantitative real-time PCR (qRT-PCR) with β-actin serving as the housekeeping gene. The iQ SYBR Green Supermix (Bio-Rad Laboratories) was used in qRT-PCR and reactions were performed in a CFX96 Thermal Cycler machine (Bio-Rad). Primer sequences for TF were: forward 50-GCC AGG AGA AAG GGG AAT-30; reverse 50-CAG TGC AAT ATA GCA TTT GCA GTA GC-30. Sequences for β-actin were: forward 50-TCA CCC ACA CTG TGC CCA TCT ACG A-30; reverse 50-CAG CGG AAC CGC TCA TTG CCA ATG G -30. Primers were purchased from Integrated DNA Technologies (Coralville, Ia). The Pfaffl method was used to calculate fold changes in TF mRNA expression levels.[27]

Statistical analysis. The results are presented as mean ± standard error of at least three separate experiments. Statistical significance was determined by analysis of variance (ANOVA) followed by Bonferroni post hoc comparison testing. Statistical significance was defined as $P < .05$. Statistical analysis was performed using Prism 6 for Mac OS X software package (GraphPad Software Inc, La Jolla, Calif).

RESULTS

TF expression in HUVECs exposed to disturbed and uniform flow in the presence or absence of thrombin. Fig 1 shows TF expression levels of sparse or confluent HUVECs exposed to mechanical stimuli for up to 4 hours compared with sparse or confluent static cultures in the presence or absence of thrombin. Previous reports[5,18,23] have demonstrated that in HUVECs exposed to flow for up to 8 hours, the peak of TF expression occurred 2 to 4 hours after initiation of flow. Fig 1, *A* shows that there was no statistical difference in TF expression level between sparse and confluent HUVECs exposed to uniform flow, although confluent HUVEC cultures exhibited significantly higher TF expression than static cultures (ANOVA, $P < .05$) at the 2-hour time point (by post hoc analysis). Fig 1, *B* shows that sparse HUVEC cultures exposed to disturbed flow exhibited higher TF expression than confluent HUVEC cultures (ANOVA, $P < .05$), at the 4-hour time point (by post hoc analysis). Both sparse and confluent HUVEC cultures exposed to disturbed flow exhibited higher TF expression than that noted in static cultures (ANOVA, $P < .05$). Fig 1, *C* shows that sparse HUVEC cultures exposed to uniform flow in the presence of thrombin exhibited higher TF expression than confluent HUVEC cultures (ANOVA, $P < .05$) at the 4-hour time point (by post hoc analysis). Both sparse and confluent HUVEC cultures exposed to uniform flow in the presence of thrombin exhibited higher TF expression than noted in static cultures in the presence of thrombin (ANOVA, $P < .05$) at both the 2-hour and 4-hour time points (by post hoc analysis; Fig 1, *D*). Sparse HUVEC cultures exposed to disturbed flow in the presence of thrombin exhibited higher TF expression than confluent HUVEC cultures (ANOVA, $P < .05$) at the 4-hour time point (by post hoc analysis; Fig 1, *C*). Both sparse and confluent HUVEC cultures exposed to disturbed flow in the presence of thrombin exhibited higher TF expression than that noted in static cultures in the presence of thrombin (ANOVA, $P < .05$) at both the 2-hour and 4-hour time points (by post hoc analysis). Sparse HUVEC cultures exposed to disturbed flow exhibited higher TF expression than cultures exposed to uniform flow (ANOVA, $P < .05$) in the absence or presence of thrombin. Confluent HUVEC cultures exposed to disturbed flow exhibited higher TF expression than

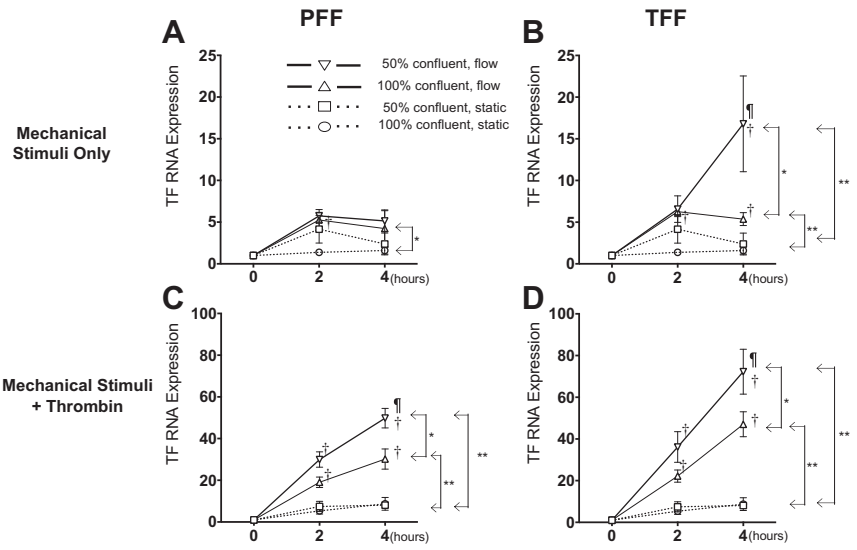

Fig 1. Tissue factor (*TF*) RNA expression in human umbilical vein endothelial cells (HUVECs) exposed to pulsatile forward flow (*PFF*) **(A** and **C)** or pulsatile to-and-fro flow (*TFF*) **(B** and **D)** in the presence **(C** and **D)** or absence **(A** and **B)** of thrombin. HUVEC cultures were subjected to 60 cpm, 14 dyne/cm^2 TFF or PFF for up to 4 hours. TF RNA expression was expressed as fold change compared with static control values at 0 hours as a baseline and the means ± standard error of three to seven experiments shown. *$P < .05$ compared 50% with 100%, **$P < .05$ compared with static, calculated by analysis of variance (ANOVA). ¶$P < .05$, compared 50% with 100%; †$P < .05$, compared with static, calculated by post hoc analysis of Bonferroni. ○, static 100% confluent; □, static 50% confluent; △, PFF or TFF 100% confluent; ▽, PFF or TFF 50% confluent.

cultures exposed to uniform flow (ANOVA, $P < .05$) only in the presence of thrombin.

Activation of PECAM-1 by either flow or thrombin stimulation. To determine the effect of uniform and disturbed flow and/or chemical stimuli on the activation of the PECAM-1 signaling pathway in sparse and confluent HUVECs, we assessed tyrosine phosphorylation of PECAM-1 (Fig 2, *A-D*). Previous studies on the modulation of thrombin-induced TF expression in HUVECs demonstrated downregulation of PECAM-1 by 5 to 30 minutes after addition of thrombin to static cultures.[18] Fig 2, *A* and *C* show that there was only minimal PECAM-1 phosphorylation in confluent HUVEC cultures exposed to either uniform or disturbed flow. Fig 2, *B* and *D* demonstrate that in sparse HUVEC cultures, PECAM-1 phosphorylation is increased by flow, but only HUVECs exposed to uniform flow demonstrated significantly higher PECAM-1 phosphorylation than that noted in static conditions.

Activation of PECAM-1 by simultaneous mechanical and chemical stimulation. Fig 2, *A* and *E* shows that PECAM-1 in static confluent HUVEC cultures was phosphorylated upon addition of thrombin and was significantly greater than confluent HUVEC cultures exposed to both flow and thrombin. Confluent HUVEC cultures exposed to disturbed flow had significantly less PECAM-1 phosphorylation compared with uniform flow (ANOVA, $P < .05$) at both the 15-minute and 30-minute time points ($P < .05$, post hoc analysis). Fig 2, *B* and *F* demonstrates that in sparse HUVEC cultures, PECAM-1 phosphorylation is significantly increased by both uniform and disturbed flow in the presence of thrombin compared with static cultures treated with thrombin (ANOVA, $P < .05$). HUVEC cultures exposed to uniform flow in the presence of thrombin exhibited higher TF expression than that exposed to disturbed flow in the presence of thrombin (ANOVA, $P < .05$).

DISCUSSION

Studies have shown that atherosclerosis occurs at a young age, with fatty streaks found in the posterior aorta and branch sites.[28] The areas of the flow divider, which are regions of high shear stress and laminar flow, are relatively protected. It is now widely accepted that regions of the vasculature where shear stress is low or oscillatory and the spatial shear stress gradient is high are more likely to develop atherosclerosis.[29-31] The lesser curvature of the aortic arch, where the time-averaged shear stress is low, and the bifurcation of aorta or common carotid artery, where flow is oscillatory, are common locations for atherosclerotic plaque.[32-35] In this study, we used TFF as a model of oscillatory, disturbed flow and PFF as a model of pulsatile uniform flow analogous to blood flow found in the straight portion of the aorta, where atherosclerotic plaques rarely develop. We demonstrated that HUVEC cultures exposed to disturbed flow exhibited lower levels of PECAM-1 activation and higher levels of TF expression than that measured in cultures exposed to unidirectional

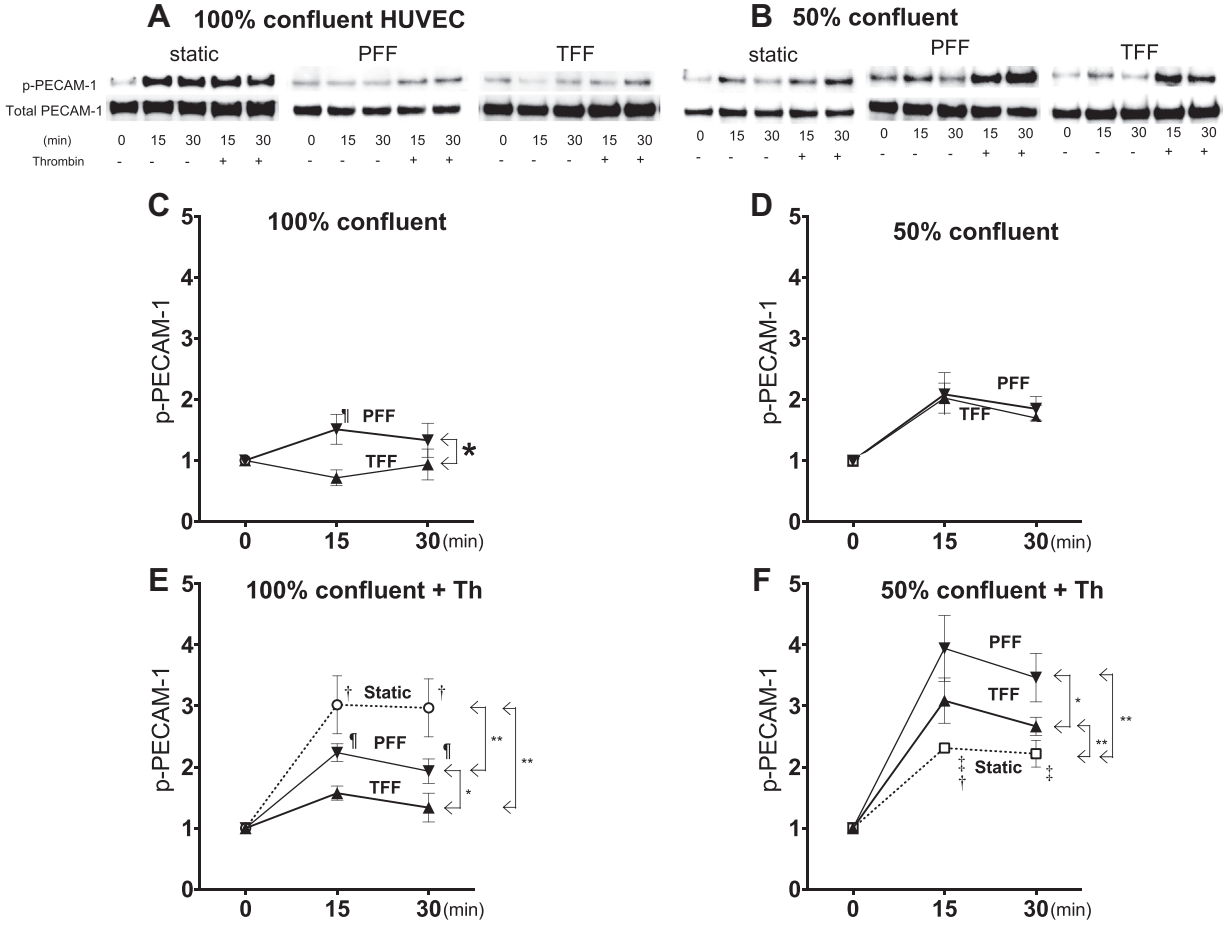

Fig 2. Platelet endothelial cell adhesion molecule-1 (*PECAM-1*) phosphorylation of human umbilical vein endothelial cells (*HUVECs*) exposed to 60 cpm, 14 dyne/cm^2 of either pulsatile forward flow (*PFF*) or pulsatile to-and-fro flow (*TFF*) for up to 30 minutes. Samples were obtained after 0, 15, or 30 minutes of mechanical and/or thrombin stimulation. **A** and **B,** Representative immunoblots of PECAM-1-immunoprecipitated samples obtained from 100% confluent HUVECs **(A)** and 50% confluent HUVECs **(B)** with anti-phosphotyrosine-specific antibody and using the anti-PECAM-1antibody as loading control. **C-F,** The densitometric means ± standard error of three to seven experiments. **C** and **D,** Phosphorylation of PECAM-1 of HUVECs exposed to PFF and TFF in 100% **(C)** and 50% **(D)** confluent HUVECs. **E** and **F,** Phosphorylation of HUVECs exposed to PFF and TFF with thrombin in 100% **(E)** and 50% **(F)** confluent HUVEC cultures. Phosphorylated-PECAM-1 is expressed as fold change compared with the baseline (time 0). *$P < .05$ comparing PFF with TFF; **$P < .05$ compared with static, calculated by analysis of variance (ANOVA). ¶$P < .05$, TFF compared with PFF; †$P < .05$, PFF compared with static; ‡$P < .05$, TFF compared with static, calculated by post hoc analysis of Bonferroni. ○, static 100% confluent; □, static 50% confluent; ▲, TFF 50% or 100% confluent; ▼, PFF 50% or 100% confluent.

pulsatile flow in either the presence or absence of chemical stimuli. Our results are consistent with previous reports that demonstrate an inverse relationship between PECAM-1 and TF expression.[18]

TF regulation by mechanical forces has been previously reported. Mazzolai et al, utilizing a cone-and-plate viscometer, demonstrated that human ECs exposed for 24 hours to oscillatory shear stress significantly increased TF mRNA and TF protein expression.[36] Lin et al reported that 12 dyne/cm^2 steady laminar shear induced the TF gene in both HUVECs and bovine aortic ECs.[37] The present study confirms our previous findings that disturbed pulsatile flow induces a greater expression of TF than uniform pulsatile flow and that there was a synergistic effect when thrombin was present.[5] In our initial report, the pulsatility of flow was much slower, the equivalent of 30 cpm (ie, 0.25 sec of forward flow alternating with 0.25 sec of either no flow or reverse flow). In addition, HUVECs were 90% to 100% confluent and were exposed to flow in FBS-free medium. We employed a more physiologic frequency of 60 cpm in the present study and, although our media had 1% FBS, we found a greater level of TF expression suggesting that the frequency of flow may be an independent variable. We also precisely regulated the

degree of confluence of HUVECs in the current study to directly investigate the role of cell-cell contact.

The mechanisms by which ECs sense and respond differently to uniform or disturbed flow is not known. PECAM-1 is a putative mechanosensor that has been shown to be tyrosine phosphorylated in ECs exposed to both steady laminar[38] and orbital laminar[22] flow, but the degree of confluence of the cultures in those studies was not precisely controlled. PECAM-1 is predominantly localized to cell-cell borders of confluent monolayers where they engage in a cis- or trans-interaction.[39] In sparse cell cultures, PECAM-1 is diffusely distributed on the cell surface.[40] Our finding that exposure of HUVECs to pulsatile flow, especially disturbed flow, had a minimal effect on PECAM-1 phosphorylation in confluent cultures and exhibited only a slight increase in sparse cultures (Fig 2, *C* and *D*) is in contrast to reports by other investigators that PECAM-1 is phosphorylated by osmotic shock[16] and nonpulsatile flow.[38] In our own studies,[22] we observed that in both bovine aortic ECs and HUVECs, cell-cell contact was required for nonpulsatile shear-induced PECAM-1 phosphorylation. This varied response of PECAM-1 phosphorylation by HUVECs exposed to pulsatile or nonpulsatile shear is not completely understood but is consistent with the known specificity of the molecular responses of ECs to distinct hemodynamic and chemical stimuli.[23,41] For instance, one of our early reports demonstrates that while laminar and pulsatile flow induced TF expression in HUVECs, orbital flow and cyclic strain did not.[23] We also demonstrated that while p38 was activated to an equal degree by either disturbed or uniform flow but not thrombin, ERK1/2 activation at 2 hours in subconfluent HUVEC cultures exposed to disturbed flow was higher than that exhibited in HUVECs exposed to uniform flow while thrombin stimulation resulted in only transient activation at 5 minutes. This differential response may also have a basis in the underlying mechanism of sensing external forces through cytoskeletal and membrane deformations. Melchoir et al[42] have reported cell-cell junction adaptation by inclination in the direction of flow. They demonstrated Akt-1 activation with disturbed flow in the region of the cell immediately downstream of the junction, which correlated with regions of high tension and an intact actin cytoskeleton. They speculated that retrograde flow resulted in destabilization of the actin cytoskeleton with reduced Akt-1 activation. Wang et al[43] have proposed a model in which cells essentially compare flow direction with an internal axis and activate cytoskeletal and signaling pathways accordingly. They suggest that an important reason why low and oscillatory disturbed flow are atherogenic is because cells fail to align in the flow direction so that many cells experience flow at high angles relative to their long axes.

In an effort to dissect out the importance of PECAM-1 as a mechanosensor and provide some mechanistic insights into the order of prioritization and cross-talk of cell signal pathways, we compared the independent and combined effect of mechanical forces and a chemical stimuli. Laminar shear-induced PECAM-1 phosphorylation leads to downregulation of Akt and endothelial NO synthase,[44] while in ECs exposed to orbital shear, MAPK and Akt signal pathways are activated independent of PECAM-1.[22] We specifically chose thrombin as the chemical stimulant because thrombin induction of TF expression has been well characterized and has been shown to involve thrombin interaction with the seven transmembrane G-protein coupled receptor (PAR-1) leading to p38 activation and an increase in the transcription factor egr-1.[26] Zhang et al reported that PECAM-1 modulates thrombin-induced expression through the PI3K/Akt pathway.[18] HUVECs transfected with antisense PECAM-1 oligonucleotides exhibited greater induction of TF mRNA compared with scrambled oligonucleotide-treated HUVECs, and this was dependent on p38 and PI3K/Akt.[18] Fig 2 demonstrates that thrombin treatment of static HUVECs exhibited greater PECAM-1 phosphorylation in confluent compared with sparse cultures. This novel finding suggests that engaged PECAM-1 at cell-cell junctions may be more amenable to activation by thrombin than diffuse PECAM-1. While this may also simply represent lower levels of expressed PAR-1 in sparse cells, there may be conformational differences between diffuse PECAM-1 and engaged PECAM-1 at cell-cell junctions that may affect tyrosine phosphorylation.[45] A difference in the regulation of cell signal pathways between sparse and confluent cells cannot also be excluded.[46]

Thrombin stimulation also appears to be a stronger activator of PECAM-1 than flow, especially in confluent static cells (comparing thrombin added to static cells in Fig 2, *E* with cells exposed to flow in Fig 2, *C*). In contrast, the combination of thrombin with pulsatile flow had distinct effects on sparse and confluent cultures. There was a significantly higher level of PECAM-1 phosphorylation in cultures exposed to combined flow and thrombin compared with either stimulus in the sparse cultures (Fig 2, *F*), but there was significantly decreased level of PECAM-1 phosphorylation in cultures exposed to combined flow and thrombin compared with thrombin alone in confluent cultures. Although the reason for the different levels of PECAM-1 phosphorylation in sparse and confluent HUVECs is not clear, reports have suggested that there is a lower expression of PECAM-1 in sparse cells.[47] However, we have previously shown no significant difference in protein levels of PECAM-1 in bovine aortic ECs, HUVECs, or murine microvascular cells that were sparse or confluent.[22] Even if there were reduced levels of PECAM-1 protein in sparse cultures, Fig 2 demonstrates that there was increased phosphorylation of PECAM-1 by flow in the sparse cultures, which indicates a significant degree of PECAM-1 activation.

CONCLUSIONS

In summary, our results suggest that cell confluence and the type of flow are critical independent factors in the induction of TF and PECAM-1 phosphorylation in

ECs in response to shear. We demonstrate two significant patterns depending on the level of confluence of HUVEC cultures. PECAM-1 in confluent HUVECs is only minimally phosphorylated by flow, especially disturbed flow, but is strongly activated by thrombin. Simultaneous mechanical and chemical stimulation results in PECAM-1 phosphorylation levels that are much lower than that seen with thrombin stimulation alone and is similar to levels exhibited with flow stimulation. In sparse HUVEC cultures, PECAM-1 is phosphorylated by both flow and thrombin, and the combined stimulation results in even higher phosphorylation. Our results are consistent with a working model that two distinct pathways are involved in PECAM-1 phosphorylation. Thrombin interacts with the PAR-1 receptor, while mechanical forces act on mechanosensors, such as PECAM-1 and/or on cytoskeletal tensegrity elements resulting in downstream cell signal cascade. In the presence of flow, the cell pathways generated by thrombin PAR-1 activation are modulated by cell signal pathways activated by flow. The responsiveness of sparse or confluent cultures to these stimuli with regard to PECAM-1 activation varies depending on the levels of diffuse PECAM-1 and engaged PECAM-1 at cell-cell junctions, but further studies will be required to elucidate the precise mechanism.

AUTHOR CONTRIBUTIONS

Conception and design: BS
Analysis and interpretation: TM, BS
Data collection: TM
Writing the article: TM, BS
Critical revision of the article: BS
Final approval of the article: BS
Statistical analysis: TM
Obtained funding: BS
Overall responsibility: BS

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